and back-titrated with hydrochloric acid, using brom thymol blue as indicator: equiv. wt., 125.

The debenzylated polymer was dialyzed and treated as in section a; yield 4.6 g. The multichain polyglutamic acid was hydrolyzed and the hydrolysate analyzed chromatographically analogously to the multichain polytyrosine. A molar ratio of glutamic acid to lysine, 16 to 1, was obtained. The intact multichain polyglutamic acid gave amino N, 0.60 (Van Slyke), and Na, 2.9, determined with a flame spectrophotometer. The multichain polyglutamic acid was desaminated analogously to the desamination of multichain poly-DL-alanine, and the product hydrolyzed as usual. The hydrolysate was analyzed chromatographically, using 1-butanol–glacial acetic acid–water (4:1:5) as the mobile phase. The chromatogram contained spots of lysine and glutamic acid, but no spots corresponding to the products appearing in the hydrolysate of desaminated polylysine.

Poly-L-lysine, Free Base.—A solution of poly-L-lysine hydrobromide, DP 18 (78 mg.), in water (5 ml.) was passed through a column of Amberlite IRA 400, and the fraction giving a negative Volhard and a positive ninhydrin test (20 ml.) was collected and dried *in vacuo*; yield, 45 mg. of a white powder.

Anal. Calcd. for poly-L-lysine (DP 18): neut. equiv., 128. Found: neut. equiv., 134, determined by titration in glacial acetic acid with perchloric acid in glacial acetic acid, using thymol blue as indicator.

The polylysine free base (DP 18) obtained could not be redissolved in water. It is soluble in glacial acetic acid, in phenol and in aqueous acids. It is insoluble in ether, dioxane, methanol, pyridine, triethylamine, tributylamine and boiling dimethylformamide. It may be precipitated from its solution in glacial acetic acid by dioxane.

Physical Measurements.—The sedimentation measurements were carried out in a Spinco ultracentrifuge Model E with a Klett optical system. The sedimentation constant was calculated as an average of the values $S = 2(x_2 - x_1)/(x_2 + x_1)\omega^2(t_2 - t_1)$, where x_1 and x_2 are the distances from the axis of rotation to the boundary at the times t_1 and t_2, respectively, and ω is the angular velocity. Final corrections were made in the customary manner to a solvent with the density and viscosity of water at 20°. For this purpose a partial specific volume of 0.72 was employed.

Diffusion measurements were carried out in a Claesson diffusion cell,[25] at 20°. A Tiselius–Longsworth apparatus[26] with a schlieren-scanning optical system was used. In the measurements made the diffusion curves were found to be practically normal. The average diffusion coefficients were derived using the formula $D = \sigma/2t$, where σ denotes the standard deviation and t the time of diffusion. The standard deviation was calculated from the first and second moments measured with an Amsler Integrator.

The refractive index of multi-poly-DL-alanyl-poly-L-lysine (b 34, p 22) in glycine buffer, pH 9.1 and ionic strength 0.1, at 20°, was measured by means of a dipping refractometer compensated to sodium light. The refractive index increment, $dn/dw = 0.182$, was calculated from the slope of linear relation between refractive index and concentration found experimentally (w is the ratio between the weight of solute and the weight of solution).

Viscosity measurements were carried out in an Ostwald viscosimeter at 20°.

(25) S. Claesson, *Nature*, **158**, 824 (1946).

(26) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).

REHOVOT, ISRAEL

CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Chromatography of Proteins. I. Cellulose Ion-exchange Adsorbents

BY ELBERT A. PETERSON AND HERBERT A. SOBER

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Ion-exchange adsorbents have been prepared from cellulose under conditions such that physical properties suitable for column chromatography are maintained. These adsorbents possess high capacity for the adsorption of proteins, yet permit elution under mild conditions. Titration curves are presented.

The remarkable success of chromatography in the separation of amino acids, lipids, sugars and many other substances has been an important factor in recent advances in biochemistry. The application of chromatography to the separation of proteins, however, has not met with the general success obtained with smaller, simpler molecules. The large size of the protein molecule prevents its penetration into the adsorbent particle, therefore only the adsorbing sites located on the exterior can be utilized. An enormous surface, as provided by very fine division, then becomes necessary for the attainment of reasonably high adsorption capacity. The instability of the protein molecule is also a factor, not only because of the severe limitations it imposes upon the choice of solvents, but also because profound configurational changes may accompany the adsorptive and desorptive processes if binding sites are sufficiently numerous and strong to compete with the multitude of weak bonds which maintain the native configuration of the protein. Even where this does not occur, too high a density of binding sites may result in adsorption so firm that the conditions required for simultaneous dissociation of all the adsorptive bonds are destructive to the integrity of the molecule.

However, progress has been made in recent years, particularly in the field of ion-exchange chromatography. Relatively stable proteins of rather low molecular weight and high isoelectric point have been chromatographed on columns of finely divided Amberlite IRC-50, a weak cation exchanger.[1] The chromatography of hemoglobin, a neutral molecule of more typical size, has been demonstrated on the same resin, but the conditions required were such as to preclude ion exchange as the adsorption mechanism.[2] Very recently the separation of four kinds of human carbon monoxide hemoglobin with IRC-50 has been reported.[3] Early work in this Laboratory achieved the frontal analysis of artificial mixtures of typical proteins on the strong cation exchanger, Dowex-50, but the capacity was very low.[4] The same resin has been used for the chroma-

(1) C. A. Zittle, *Advances in Enzymology*, **14**, 319 (1953); E. Margoliash, *Nature*, **170**, 1014 (1952); *Biochem. J.*, **56**, 529, 535 (1953); C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953); C. H. W. Hirs, *ibid.*, **205**, 93 (1953); C. F. Crampton, S. Moore and W. H. Stein, *ibid.*, **215**, 787 (1955).

(2) N. K. Boardman and S. M. Partridge, *Nature*, **171**, 208 (1953); *Biochem. J.*, **59**, 543 (1955).

(3) H. K. Prins and T. H. J. Huisman, *Nature*, **175**, 903 (1955).

(4) H. A. Sober, G. Kegeles and F. J. Gutter, THIS JOURNAL, **74**, 2734 (1952); *Science*, **110**, 564 (1949).

tography of prostatic phosphatase,[5] and the application of an anion exchanger, Dowex-2, to the fractionation of human serum proteins has very recently been reported.[6] The use of inorganic gels such as calcium phosphate has been promising in the chromatography of typical proteins,[7] but adsorbents with greater chemical and physical stability, allowing a wider selection of eluents, and with better defined ionizing groups are to be desired.

In the following pages the preparation and properties of new adsorbents derived from cellulose, having these characteristics as well as relatively large capacities for the adsorption of proteins, will be described. The effectiveness of such adsorbents containing carboxymethyl (CM–), diethylaminoethyl (DEAE–) and phosphate (P–) groups in the chromatographic purification of enzymes and other proteins has been reported in preliminary communications from this Laboratory.[8] A companion paper demonstrates the resolution achieved in the separation of serum proteins on DEAE–cellulose, an anion exchanger.[9] Further information concerning the use of these adsorbents in the chromatography of enzyme preparations will be published at a later date.

Another adsorbent prepared in this Laboratory, ECTEOLA–cellulose,[10] containing basic groups derived from triethanolamine bound to cellulose through reaction with epichlorohydrin, was found to be less promising in protein adsorption experiments but will be described here because of its exceptional merit in the chromatography of nucleic acids, as shown by work carried out in other laboratories.[11]

A number of cellulose ion exchangers have appeared in the literature, including oxycellulose,[12] cellulose succinic half ester,[13] and a variety of treated cotton fabrics.[14] Although oxycellulose has been used for the purification of ACTH oligopeptide preparations by batch adsorption,[15] no attempt to employ materials of this type for the adsorption of proteins has been reported. In several instances, however, the adsorption of enzymes by cellulose, itself, has been observed and utilized for purification.[16] Such adsorption can probably be attributed to a very small carboxyl content normally present in cellulose.

General Considerations.—Alpha cellulose offered particular promise as a support for ionizing groups intended to bind proteins because of its hydrophilic nature and enormous surface. It was readily available at low cost, and simple modifications of a reaction extensively used in the industrial manufacture of cellulose derivatives provided the means of attaching a variety of ionizable groups. The preparation of ion exchangers of this type is, however, subject to a limitation arising from the nature of the cross-linking bonds in cellulose. Hydrogen bonding between the hydroxyl groups is responsible for the insolubility of that material, and the attachment of small amounts of any substituent to the chain tends to increase swelling in water by interfering with hydrogen bonding. A larger number of groups will cause the cellulose derivative to become water-soluble, like the carboxymethylcellulose of industry. Although a certain amount of swelling is advantageous in protein chromatography in that greater accessibility to the binding sites is afforded, there is a concomitant increase in resistance to the flow of aqueous solutions which is of considerable importance when the material is to be used in adsorption columns. In the procedures to be described the degree of incorporation is controlled to keep the physical properties of the product within the range useful for chromatographic purposes. It has been regarded as essential to avoid conditions which might solubilize or disperse the product, even temporarily, for in those cases where reprecipitation was possible a gelatinous material having extremely high resistance to the flow of aqueous solutions was obtained. Such an adsorbent may be useful in batch operations when sedimentation can be employed for separation of the phases, but it is ill-suited to column chromatography or batch filtration. Although insoluble preparations of both CM– and DEAE–cellulose containing 2 meq. per gram have been obtained, products containing much more than 1 meq. per gram had a tendency to be gelatinous, with a high resistance to the flow of water. This limitation on the number of groups incorporated proved to be not serious, for the capacity for protein adsorption was very high, on a mass basis, even when the acid–base capacity of the adsorbent was of the order of 1 meq. per gram. Since multiple-site binding is undoubtedly involved in the protein–adsorbent combination, a low density of binding sites on the adsorbent is advantageous (provided that the capacity is adequate) in that it must contribute toward ease of desorption of polyelectrolytes. A low buffering capacity (desirable in gradient elution chromatography) and a low capacity for the adsorption of small electrolytes are additional favorable consequences.

The cellulose ion exchangers described here have all been prepared by the reaction of a chloro compound with cellulose which has been allowed to swell in strong alkali. In general, the number of

(5) H. G. Boman, *Biochim. Biophys. Acta*, **16**, 245 (1955).

(6) H. G. Boman, *Nature*, **175**, 898 (1955).

(7) K. Agner, *Biochem. J.*, **32**, 1702 (1938); S. M. Swingle and A. Tiselius, *ibid.*, **48**, 171 (1951); A. Tiselius, *Arkiv Kemi*, **7**, 443 (1954).

(8) H. A. Sober and E. A. Peterson, THIS JOURNAL, **76**, 1711 (1954); E. A. Peterson and H. A. Sober, *Federation Proc.*, **13**, 273 (1954); H. A. Sober and E. A. Peterson, Abstracts 126th National Meeting, Am. Chem. Soc., 90-C, Sept., 1954.

(9) H. A. Sober, F. J. Gutter, M. M. Wyckoff and E. A. Peterson, THIS JOURNAL, **78**, 756 (1956).

(10) The intended reactions involved ether formation between the hydroxyl groups of triethanolamine and the epoxide ring of epichlorohydrin, as well as between the halogen function of the latter and the NaO–groups of the sodium cellulose. However, since other reactions are possible (including polymerization) the structure of the attached groups cannot be specified, and the adsorbent has been designated ECTEOLA–cellulose to identify the reactants.

(11) A. Bendich, J. R. Fresco, H. S. Rosenkranz and S. M. Beiser, THIS JOURNAL, **77**, 3671 (1955); D. F. Bradley and A. Rich, Abstracts 128th National Meeting, Am. Chem. Soc., 3-C, Sept., 1955.

(12) E. C. Yackel and W. O. Kenyon, THIS JOURNAL, **64**, 121 (1942).

(13) F. C. McIntire and J. R. Schenck, *ibid.*, **70**, 1193 (1948).

(14) C. L. Hoffpauir and J. D. Guthrie, *Textile Research J.*, **20**, 617 (1950); W. A. Reeves and J. D. Guthrie, *ibid.*, **23**, 522 (1953); L. M. Soffer and E. Carpenter, *ibid.*, **24**, 847 (1955).

(15) E. B. Astwood, M. S. Raben, R. W. Payne and A. B. Grady, THIS JOURNAL, **73**, 2969 (1951).

(16) H. Tauber, *J. Biol. Chem.*, **113**, 753 (1936); R. W. Payne, M. S. Raben and E. B. Astwood, *ibid.*, **187**, 719 (1950); R. O. Hurst and G. C. Butler, *ibid.*, **193**, 91 (1951).

groups attached per gram of dry product was approximately a linear function of the amount of chloro compound used, provided sufficient alkali was present, and so long as the incorporation did not greatly exceed 1 mmole per gram. The degree of reaction appeared to be largely independent of the particle size of the cellulose employed, at least over the range of 100 mesh to finer than 325 mesh. Good reproducibility with respect to number of groups attached and gross physical properties was obtained under the conditions described.[17] If, however, the incorporation were carried to a point approaching that at which gels were obtained, poor reproducibility of both incorporation and physical properties was observed.

Experimental

Cellulose.—The cation exchangers, CM- and P-cellulose, were prepared from Whatman ashless standard cellulose powder,[18] which has a cotton origin. In preparations of this type, wood cellulose offered little advantage with respect to reactivity, whereas the white color of the Whatman product was highly desirable. The anion exchanger, DEAE-cellulose, was preferably made from wood cellulose, for the reactivity of the latter was about twice that of cotton cellulose in this reaction. Polycel,[19] a wood cellulose, was initially employed in preparations of DEAE-cellulose. These products possessed excellent properties for the column chromatography of proteins, and considerable use was made of them[8,9] before it became known that Polycel was no longer being manufactured. Subsequent experiments have employed Solka-Floc, another wood cellulose of high purity.[20] Of the several grades available, SW-A, SW-B, and BW have been tested in the preparation of DEAE-cellulose. All were comparable to Polycel in reactivity. Products made from the SW-A and BW grades, however, exhibited a greater tendency to shed "fines" on washing and a somewhat greater resistance to the flow of aqueous solutions which was particularly marked in strong alkali. Products made from the SW-B grade were intermediate in these respects. Adequate amounts of all the particle sizes used in these experiments were obtained by sieving commercial 40 or 100 mesh material.

2-Chlorotriethylamine Hydrochloride.—The commercially available material (Eastman Kodak, P6436) was recrystallized from absolute methanol to remove highly colored impurities.

Other Materials.—Chloroacetic acid, epichlorohydrin and triethanolamine (2,2',2"-nitrilotriethanol) were white label products of the Eastman Kodak Company and were used without further purification.

Preparation of DEAE-SF.—A solution of 40 g. of NaOH in 170 ml. of water was stirred[21] into 60 g. of Solka-Floc. The mixture, dry in appearance, was allowed to stand in an ice-bath for 30 minutes,[22] with occasional stirring. A solution of 35 g. of recrystallized 2-chlorotriethylamine hydrochloride in 45 ml. of water was added in several portions. After thorough blending, the mixture was immersed in an 80–85° oil-bath for 35 minutes and stirred occasionally. The product was cooled in an ice-bath and 250 ml. of 2 *M* NaCl[23] was added in several portions, care being taken to achieve complete mixing.

The resulting thick suspension was filtered with gentle suction in a 5-inch coarse sintered glass funnel, and the filter cake was washed with 1 *N* NaOH until the emerging filtrate was no longer deeply colored. The product was then resuspended on the filter in sufficient 1 *N* HCl (about 350 ml.) to make a strongly acid suspension, and this was immediately filtered. Successive washing with 250-ml. portions of 1 *N* NaOH, 1 *N* HCl and 1 *N* NaOH followed. The cake was tamped down and pulled dry to the cracking point between washings but was not rinsed with water. Finally, the cake was again suspended in 250 ml. of 1 *N* NaOH, then transferred to a large vessel and diluted with water to 3 liters. After standing overnight, the cloudy supernatant liquid was decanted, and the sediment was washed 5 or 6 times by decantation to remove particles that would not settle.[24] The final supernatant liquid was almost clear and was free of alkali. The sediment was filtered to remove as much water as possible, then washed with about 500 ml. of ethanol in 3 portions, the last one absolute ethanol. As much of the alcohol as possible was drawn off on the filter, then the partially dried product was abraded to a powder. The remaining alcohol was evaporated *in vacuo*,[25] with a sintered glass disc inserted at the mouth of the flask to prevent the escape of the light powder into the cold trap and pump. When cellulose finer than 325 mesh was used, about 45 g. of adsorbent containing approximately 1.4% nitrogen (1.0 meq. per gram) was obtained. With coarser cellulose the yields were somewhat greater.

Preparation of CM-W.—A solution of 90 g. of NaOH in 200 ml. of water was stirred into 60 g. of Whatman cellulose powder (sieved to remove material coarser than 325 mesh) to form a stiff, friable mass.[21] The flask was then immersed in an ice-bath for 30 minutes,[22] and the contents were stirred occasionally. A solution of 30 g. of chloroacetic acid in 40 ml. of water was added in 3 or 4 portions and mixed well after each addition. The soft, doughy mass was then heated in a 70° oil-bath for 20 minutes, with occasional stirring. After removal from the oil-bath, the bright yellow product was cooled in an ice-bath, and 500 ml. of 10% acetic acid (or an equivalent amount of dilute HCl) was added in several portions. The suspension, which was still strongly alkaline, was then diluted to 2 liters with water and allowed to settle. After careful decantation of the cloudy yellow supernatant fluid, the sediment was washed repeatedly in a similar manner to remove particles which would not sediment, as well as color and alkali.[24] About 10 ml. of glacial acetic acid was then added to the diluted suspension to make it just acid to congo red, and after settling and decantation of the supernatant liquid, a slurry of the sediment was poured into a 5 inch coarse sintered glass funnel and washed with water until the filtrate was no longer acid. The cake was pulled as dry as possible on the filter, then washed with ethanol and dried in the manner described for the preparation of DEAE-SF. About 45 g. of white powder containing 0.7 meq. of acidic groups per gram was obtained.

Preparation of P-W.—A solution of 75 g. of NaOH in 185 ml. of water was stirred into 50 g. of Whatman cellu-

(17) In six preparations of DEAE-SF, following the same procedure, the average deviation from the mean nitrogen content was 2%.

(18) H. Reeve Angel and Company, Inc., 52 Duane Street, New York 7, New York.

(19) The Polycel was a gift of the Industrial Chemical Sales Division of the West Virginia Pulp and Paper Company, Covington, Virginia.

(20) The Solka-Floc was generously provided by the Brown Company, 500 Fifth Avenue, New York 36, N. Y.

(21) Since the volume of the added liquid was restricted to increase the efficiency of the reaction with cellulose, uniform distribution of the alkali among the particles was difficult. Therefore, the alkali was added in several small portions and the mixture was stirred well after each addition to break up the wet lumps and coat the fragments with relatively dry material.

(22) Because the mercerization of cellulose proceeds at lower concentrations of alkali when the temperature is low, the swelling process was carried out in an ice-bath to promote penetration of the particles while at the same time diminishing reaction of the strongly alkaline cellulose with oxygen.

(23) The presence of NaCl controlled swelling and facilitated filtration, which was otherwise sometimes extremely difficult under the strongly alkaline conditions required for the removal of colored side products from the anion exchangers.

(24) Because of variations in settling rate under different conditions of *p*H and ionic strength, as well as variations among the different kinds of cellulose used, a fixed settling time cannot be specified. Usually 1–2 hours was sufficient. At some point in the procedure the small amount of coarse, aggregated material present in the suspension was eliminated by careful decantation of the dilute suspension after it had been permitted to settle for a few minutes.

(25) All of the adsorbents prepared in this study were dried before use in order to facilitate characterization and storage. It is only resonable to assume that the wet products were suitable for use at any stage after the washing process, and storage as the alcohol-damp powder may be most convenient, at least in the case of the anion exchangers. Partial esterification might be expected if the CM- and P-celluloses are stored for long periods in alcohol. The vacuum method of removing the alcohol was adopted when initial attempts to air-dry CM-cellulose preparations on a filter, after washing with alcohol and ether, yielded hard, lumpy material.

lose and the well mixed,[21] friable mass was allowed to stand in an ice-bath for 30 minutes.[22] Then 330 ml. of water was added slowly. The suspension was stirred mechanically at a slow rate, with the bottom of the flask immersed in an ice-bath. After about 15 minutes the dropwise addition of 80 ml. of an ether solution containing 40 ml. of reagent grade $POCl_3$ was begun. Throughout the addition, which was completed in 50 minutes, the temperature of the suspension was maintained at 25–30°. Its final *p*H was approximately 6.

After a few minutes the suspension was diluted with water to 2 liters in a large precipitation jar and allowed to settle overnight. The sediment was washed repeatedly by decantation[24] until an almost clear supernatant liquid was obtained. It was then filtered, washed with alcohol and dried as described for the preparation of DEAE–SF. The product was a white powder weighing 38 g. and containing 1.9% phosphorus (0.6 mmole phosphate per gram).

Preparation of ECTEOLA–SF.—A solution of 60 g. of NaOH in 150 ml. of water was stirred into 60 g. of Solka-Floc and the well blended mixture[21] was immersed in an ice-bath for 30 minutes.[22] Then a mixture of 35 ml. of triethanolamine and 60 ml. of epichlorohydrin was added in several portions. The friable mass was stirred for several minutes to assure thorough mixing and penetration. It was then immersed in a 75–80° oil-bath for 30 minutes and stirred at intervals.

Approximately 10 minutes after immersion an exothermic reaction suddenly became manifest by a sharp rise in internal temperature to well over 100°, accompanied by a rapid evolution of vapor. This reaction subsided very quickly, leaving a gummy amber mass. At the end of the heating period the contents of the flask were cooled in an ice-bath, and 250 ml. of 2 *M* NaCl[23] was added in portions, stirring well after each addition to obtain a smooth suspension. The product was then filtered, washed and dried in the manner described for the preparation of DEAE–SF. About 60 g. of adsorbent containing 0.25 to 0.30 meq. of basic nitrogen per gram was obtained.

In one experiment the mixture of triethanolamine, epichlorohydrin and NaOH–cellulose was allowed to stand overnight at room temperature before heating. A very slow and moderate rise in temperature occurred spontaneously during the first two hours of standing, and there was no evidence of further reaction when a portion of the mixture was heated the next day. After washing and drying, identical nitrogen contents (0.42 meq. basic nitrogen per gram) and titration curves were obtained from the heated and unheated products. Their adsorption behavior has not yet been evaluated.

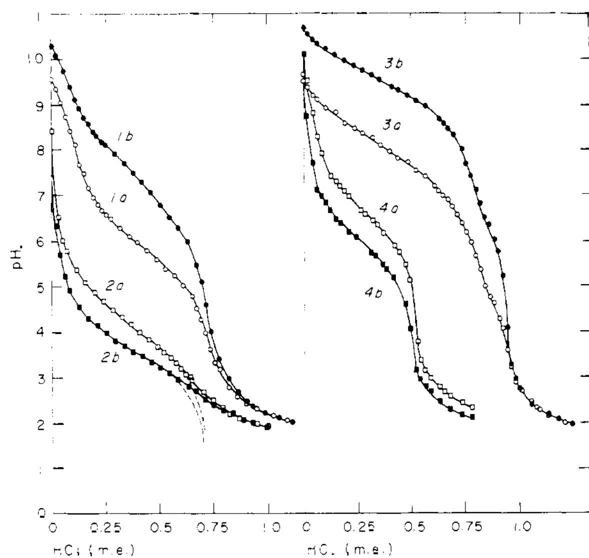

Figure 1.—Titration curves of four cellulose ion exchangers: 1, ECTEOLA–SF; 2, CM–W; 3, DEAE–SF; 4, P–W. (a) Curves obtained in water alone, (b) those obtained in 0.5 *M* NaCl. Broken lines are corrected for water blank. The small shoulder on the acid side of the DEAE–SF was apparently due to bicarbonate formation during the washing procedure. It was reduced but not eliminated by alkali treatment followed by washing with boiled water. Estimations of the *pK'* in this case were based upon the symmetrical portion of the rest of the curve.

Acid or Base Content.—The extent of incorporation of ionizing groups was determined readily in the DEAE–, ECTEOLA– and P–celluloses by nitrogen or phosphorus analysis, as well as by potentiometric titration.[26] Where it was available, the elemental analysis was taken as the basis for calculating the content of such groups, but agreement between the two methods was good. The carboxyl content of the CM–celluloses was obtained solely by titration with HCl after the addition of a small excess of NaOH. The magnetically stirred suspensions reached a steady *p*H value very rapidly after each addition of acid, permitting the titrations to be carried out as for homogeneous systems. This was further substantiated by the fact that points obtained after a 20-hour equilibration of CM–W or DEAE–SF with measured amounts of acid fell upon curves obtained by rapid titration. An ordinary glass electrode was employed without applying a sodium correction, but the procedure provided quickly a valid estimate of the ionizable groups attached to the cellulose and a close approximation of the apparent dissociation constant.

Titration Curves.—Representative titration curves appear in Fig. 1. In every case, titration in water alone yielded

TABLE I

EFFECTS OF TYPE OF CELLULOSE AND NUMBER OF GROUPS ON THE *pK'* OF CELLULOSE ION EXCHANGERS

Cellulose[a]	Ionizing groups, mmole/g.	*pK'* in water[b]	*pK'* in 0.5 *M* NaCl
DEAE–cellulose			
Solka-Floc SW-A, 325–	0.45	7.5	9.5
Solka-Floc SW-A, 325–	0.99	8.0	9.5
Solka-Floc SW-A, 325–	1.34	8.5	9.5
Solka-Floc BW, 230–325	0.99	8.0	9.5
Solka-Floc BW, 100–230	.97	8.1	9.5
Solka-Floc SW-B, 325	.94	8.0	9.5
Solka-Floc SW-B, 100–230	.90	7.6	9.5
Polycel, 270–325	.78	7.5	..
Polycel, 325–	.92	7.5	9.5
Polycel, 325–	1.03	7.5	..
Polycel, 325–	1.98	8.0	9.4
Whatman, 325–	0.18	6.5	..
Whatman, 325–	.31	6.5	..
ECTEOLA–cellulose			
Solka-Floc SW-A, 325–	.29	5.9	7.4
Solka-Floc BW, 325–	.25	6.0	7.6
Solka-Floc BW, 325–	.42	5.8	7.5
CM–cellulose			
Whatman, 325–	.75	4.2	3.5
Whatman, 325–	1.10	3.8	..
Whatman, 325–	1.57	3.8	..
Solka-Floc BW, 325–	0.59	4.3	..
Solka-Floc BW, 325–	.67	4.3	3.7
P–cellulose			
Whatman, 325–	.60	6.7	6.0
Whatman, 325–	.50	6.8	..

[a] Figures indicate mesh size. Finer than 325 mesh is designated by 325–. [b] Since NaCl was formed during the titration of CM– and P–celluloses, those titrations were not carried out in salt-free water. The concentration did not exceed 0.03 *M* at the mid-point of the titration in most cases, however.

(26) We are indebted to Mr. R. J. Koegel and his staff for the determinations of nitrogen and phosphorus and to Mrs. Mary M. Wyckoff for technical assistance in titrating the adsorbents.

a pK' corresponding to much weaker acidity or basicity than that exhibited by similar groups in free solution.[27] However, the addition of NaCl at the start of the titration resulted in pK' values which were closer to those expected. This phenomenon is characteristic of insoluble polyelectrolytes and appears to be a reflection of the relative inaccessibility of interior groups to the titrating agent.[28] The data of Table I demonstrate this effect and also differences in pK' arising from the kind of cellulose used and the number of groups incorporated. Thus, among the DEAE-celluloses, values obtained in water alone ranged from 6.5 (cotton cellulose, 0.18 meq. per g.) to 8.5 (wood cellulose, 1.34 meq. per g.), whereas with a given cellulose and a given number of attached groups reproducibility within 0.2 pH unit was obtained. The pK' values obtained by titration in the presence of 0.5 M NaCl, however, were characteristic only of the groups involved and were not affected by the kind of cellulose and the number of groups attached. Presumably, the accessibility of the ionizing sites to hydrogen ions is dependent to some extent upon the factors mentioned. That accessibility of the sites to protein is similarly affected is suggested by the following adsorption experiments.

Protein Adsorption Capacity.—The specific adsorption values listed in Table II represent the amount of protein adsorbed by 100 mg. of adsorbent suspended in a buffer in which the protein concentration was gradually increased to 2 mg. per ml. over a period of about two hours by the addition of a concentrated solution at a constant rate. At higher concentrations of protein additional adsorption occurred, but in this region the slope of the isotherm was much lower.[31] Since a measure of the adsorption capacity was desired, conditions of pH and salt concentration were selected under which desorption of the protein was negligible. The table demonstrates the very high capacity of these adsorbents for bovine plasma albumin and for carbon monoxide hemoglobin of the horse, as well as the influence of the kind of cellulose and the number of incorporated groups on the adsorption capacity. DEAE-cellulose prepared from Polycel possessed an adsorption capacity for crystalline bovine plasma albumin significantly lower than that of a similar adsorbent prepared from Solka-Floc SW-A or BW, whereas Solka-Floc SW-B yielded a material with intermediate adsorption capacity. The capacity increased rapidly with increasing incorporation of ionizing groups beyond the range compatible with physical properties suitable for column chromatography, and it is reasonable to believe that gelatinous adsorbents having much higher capacities could be prepared for special purposes. The experiments with DEAE-cellulose indicated that adsorbents made from 100-230 mesh cellulose possessed the same adsorption capacity as those made from material finer than 325 mesh.

TABLE II

ADSORPTION CAPACITIES OF CELLULOSE ION EXCHANGERS

Adsorbent	Cellulose[a]	Ionizing groups meq./g.	Specific adsorption[b]
Anion exchangers[c]			
DEAE-SF	Solka-Floc SW-A, 325-	0.45	16
DEAE-SF	Solka-Floc SW-A, 325-	0.96	75
DEAE-SF	Solka-Floc SW-A, 325-	1.34	122
DEAE-SF	Solka-Floc BW, 100-230	0.97	76
DEAE-SF	Solka-Floc SW-B, 325-	.94	66
DEAE-Pol.	Polycel, 270-325	.10	7
DEAE-Pol.	Polycel, 270-325	.78	36
DEAE-Pol.	Polycel, 325-	.91	54
ECTEOLA-SF	Solka-Floc SW-A, 325-	.29	5
Cation exchangers[d]			
CM-W	Whatman, 325-	.73	98
CM-SF	Solka-Floc BW, 325-	.67	98
P-W	Whatman, 325-	2 × 0.60	93

[a] Figures indicate mesh size. Finer than 325 mesh is designated by 325−. [b] Expressed as mg. protein adsorbed per 100 mg. adsorbent when final protein concentration is 2 mg. per ml. [c] Tested with crystalline bovine plasma albumin[29] in 0.01 M sodium phosphate at pH 7.0. [d] Tested with horse carbon monoxide hemoglobin[30] in 0.01 M sodium phosphate at pH 6.0.

The adsorbed albumin could be eluted quantitatively with 0.05 M sodium phosphate at pH 5.0.

The adsorption capacities of the cation exchangers were evaluated with solutions of horse carbon monoxide hemoglobin. The data available in Table II show that both the carboxylated and the phosphorylated celluloses adsorbed a quantity of this protein approximately equal to their own weight under the conditions indicated. It could be eluted quantitatively with 0.02 M phosphate buffer at pH 7.5. CM-celluloses made from Whatman powder and from Solka-Floc displayed identical capacities. It should be mentioned that a value of 180 mg. of protein per 100 mg. of adsorbent was obtained when a narrow ammonium sulfate subfraction of crystalline CO-hemoglobin was adsorbed on CM-W. There is reason to believe that this is a reflection of heterogeneity of crystalline CO-hemoglobin, since chromatographic experiments performed in this Laboratory have indicated the presence of several components in the usual preparation which exhibited markedly different affinities for CM-cellulose although their adsorption spectra were nearly identical.[32]

Physical Characteristics and Stability.—Columns were prepared from slurries of these adsorbents, adjusted to the proper pH and molarity of buffer, by allowing the material to settle in glass tubes fitted with coarse sintered glass bottoms under flow conditions induced by gravity alone, then applying air pressure (10 p.s.i.) to compact the adsorbent. Columns prepared in this manner possessed the ability to hold the buffer level at the top of the adsorbent against the pull of gravity. Flow rates suitable for chromatographic purposes were attainable in columns as long as 40 cm. by means of an adjustable hydrostatic head. In the case of CM- and p-celluloses prepared from Whatman powder the head required for a flow rate of 5 ml. per cm.^{2} per hour was generally less than the height of the column, itself. DEAE-cellulose prepared from Solka-Floc finer than 325 mesh required a head equal to 2-3 times the column height to attain a similar rate. Since the particle size did not appear to affect the adsorption capacity markedly, the use of coarser cellulose to obtain higher flow rates is suggested. There was little or no change in the dimensions of the columns in passing through a 4-8 pH range, but very high salt concentrations caused some shrinkage in the cation exchangers. These comments are based upon experience with sodium phosphate and chloride solutions. Other ions may alter the properties of these adsorbents materially.

These exchangers could be used repeatedly and suffered no apparent deterioration upon months of storage in a cold room in contact with aqueous solutions ranging from 0.05 M NaH_2PO_4 to 1 N NaOH.[33] Prolonged exposure to solutions more acid than pH 4 was avoided, although it is known that brief contact with 1 N HCl is not significantly harmful. After each use the anion exchangers were treated with an equal volume of 1 N NaOH to remove any residual protein, then washed with water to remove the alkali. A mixture of 0.5 M NaCl-0.5 N NaOH was used in the case of CM-cellulose to control the swelling effect of strong alkali on this adsorbent.

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(27) Glycolic acid, diethylaminoethanol, and triethanolamine were titrated in water in the same manner, giving pK' values of 3.7, 9.9 and 7.8, respectively. The corresponding values obtained in 0.5 M NaCl were 3.6, 10.0 and 8.0.

(28) H. P. Gregor and J. I. Bregman, THIS JOURNAL, **70**, 2370 (1948); R. Kunin and R. J. Myers, *ibid.*, **69**, 2874 (1947); P. Alexander and J. A. Kitchener, *Textile Research J.*, **20**, 203 (1950).

(29) Armour Laboratories, Chicago, Illinois.

(30) G. Kegeles and F. J. Gutter, THIS JOURNAL, **73**, 3770 (1951).

(31) A more complete report on adsorption isotherms will be published at a later date.

(32) Unpublished work.

(33) Phosphorylated cellulose is a probable exception. It was not subjected to the conditions described, but susceptibility of its ester bond to acid or alkaline hydrolysis can be expected.